

# Genetic Modulation of PPAR $\gamma$ Phosphorylation Regulates Insulin Sensitivity

Shamina M. Rangwala,<sup>1,3</sup> Ben Rhoades,<sup>1,3</sup>  
Jennifer S. Shapiro,<sup>1,3</sup> A. Sophie Rich,<sup>1,3</sup>  
Jason K. Kim,<sup>4</sup> Gerald I. Shulman,<sup>4</sup>  
Klaus H. Kaestner,<sup>2,3</sup> and Mitchell A. Lazar<sup>1,2,3,\*</sup>

<sup>1</sup>Division of Endocrinology, Diabetes,  
and Metabolism

Department of Medicine

<sup>2</sup>Department of Genetics

<sup>3</sup>The Penn Diabetes Center

University of Pennsylvania School of Medicine  
Philadelphia, Pennsylvania 19104

<sup>4</sup>Departments of Internal Medicine, Cell Biology,  
and Cellular and Molecular Physiology and  
Howard Hughes Medical Institute  
Yale University School of Medicine  
New Haven, Connecticut 06510

## Summary

Obesity-associated diabetes is epidemic in industrialized societies. The nuclear receptor *peroxisome proliferator-activated receptor  $\gamma$*  (PPAR $\gamma$ ) is highly expressed in adipose tissue and the presumed molecular target for antidiabetic thiazolidinedione drugs that reverse insulin resistance but also promote weight gain. Phosphorylation reduces the activity of PPAR $\gamma$  in vitro, but physiological relevance has not been demonstrated. We have studied mice homozygous for a mutation (S112A) that prevents PPAR $\gamma$  phosphorylation. Surprisingly, the weights and adipose mass of PPAR $\gamma$ -S112A mice are not greater than wild-type. Remarkably, however, genetic prevention of PPAR $\gamma$  phosphorylation preserves insulin sensitivity in the setting of diet-induced obesity. Underlying this protection are smaller fat cells, elevated serum adiponectin, and reduced free fatty acid levels. Thus, the phosphorylation state of PPAR $\gamma$  modulates insulin sensitivity. Compounds that prevent PPAR $\gamma$  phosphorylation or ligands that induce the conformation of nonphosphorylated PPAR $\gamma$  may selectively enhance insulin sensitivity without increasing body weight.

## Introduction

Obesity has become a major public health concern in most industrialized societies (Friedman, 2003). Of particular concern is the strong correlation between adiposity and type 2 diabetes, as well as the “metabolic syndrome” associated with resistance to the actions of insulin. Adipose tissue is now recognized to be an endocrine organ, and fat-derived circulating factors such as free fatty acids (FFA) and proteins such as leptin, adiponectin, resistin, TNF $\alpha$ , and IL-6 all may contribute to organismal insulin sensitivity (Ahima and Flier, 2000). A member of the nuclear hormone receptor superfamily, *peroxisome proliferator-activated receptor  $\gamma$*  (PPAR $\gamma$ ),

is the master regulator of adipogenesis in vitro and in vivo (Rosen and Spiegelman, 2001). Thiazolidinedione (TZD) ligands for PPAR $\gamma$  effectively ameliorate insulin resistance and are used clinically to improve type 2 diabetes (Olefsky and Sirtiel, 2000).

The mechanism by which TZD-mediated activation of PPAR $\gamma$  improves insulin sensitivity in diabetes is unclear. In rodents and people, PPAR $\gamma$  is most abundant in adipose tissue (Chawla et al., 1994; Tontonoz et al., 1994), and the insulin sensitivity caused by treatment with PPAR $\gamma$  ligands occurs despite weight gain, which generally predisposes to insulin resistance (Reginato and Lazar, 1999). It has been difficult to determine from genetic models whether PPAR $\gamma$  has effects on insulin sensitivity that are independent of changes in adiposity. Targeted ablation of the PPAR $\gamma$  gene locus in mice results in early embryonic lethality (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999). Heterozygous PPAR $\gamma$  null mice have improved insulin tolerance and have been reported to be protected from diet-induced obesity and insulin resistance, although this has not been a consistent finding (Kubota et al., 1999; Miles et al., 2000, 2003; Yamauchi et al., 2001a). Humans with a dominant-negative PPAR $\gamma$  allele are severely insulin resistant but also lipodystrophic (Savage et al., 2003).

PPAR $\gamma$  activity is regulated by MAP kinase phosphorylation of serine 112, which reduces its transcriptional activity (Adams et al., 1997; Camp and Tafuri, 1997; Hu et al., 1996). The implications of this posttranslational modification of PPAR $\gamma$  activity are significant, as various growth factors and cytokines could affect transcriptional activation of numerous genes involved in lipid metabolism via this pathway. However, the biological significance of PPAR $\gamma$  phosphorylation is not well understood. Interestingly, a mutation that increases PPAR $\gamma$  activity by preventing phosphorylation has been found in obese humans (Ristow et al., 1998). These individuals were reported to be morbidly obese with greater than expected insulin sensitivity.

To better understand the biological function of PPAR $\gamma$  phosphorylation, we have created a mouse in which the serine 112 codon has been changed to alanine. The resulting mice do not manifest increased adiposity on normal or high-fat chow, but are protected from insulin resistance in the setting of diet-induced obesity. Remarkably, the genetic prevention of PPAR $\gamma$  phosphorylation preserves insulin sensitivity in the setting of diet-induced obesity. Underlying this protection are changes in fat cell size, gene expression, and secreted factors that are likely to contribute to the insulin sensitivity.

## Results and Discussion

Mice with a serine-to-alanine substitution at codon 112 of PPAR $\gamma$ 2 were generated by homologous recombination (Figures 1A–1D). The resulting PPAR $\gamma$ -S112A mutant has been shown to be nonphosphorylatable and more active than wild-type PPAR $\gamma$  (Adams et al., 1997;

\*Correspondence: lazarm@mail.med.upenn.edu

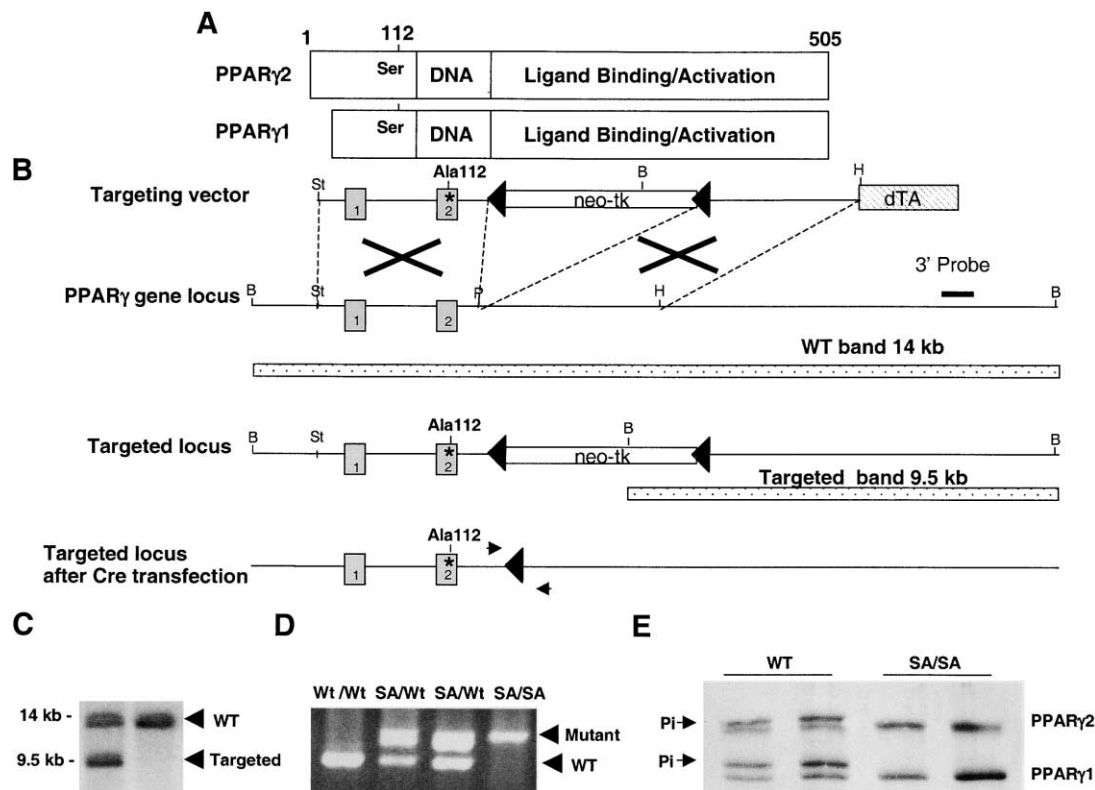


Figure 1. Generation of PPAR $\gamma$ -S112A Mice

(A) Schematic representation of PPAR $\gamma$ .

(B) Targeting strategy for PPAR $\gamma$ -S112A.

(C) ES cells with PPAR $\gamma$ -S112A mutation. Southern analysis using the 3' probe shown in (B).

(D) PCR around remaining lox P site in tail DNA of mutant and wild-type mice.

(E) PPAR $\gamma$  protein in white adipose tissue. Immunoblot analysis of PPAR $\gamma$  after SDS-PAGE to separate PPAR $\gamma$ 1 and  $\gamma$ 2 isoforms, and phosphorylated forms (Shao et al., 1998). Equal loading was confirmed by Ponceau S staining (not shown). Densitometry of multiple immunoblots revealed no significant difference in the total amount of PPAR $\gamma$ 1 or PPAR $\gamma$ 2 protein between wild-type and PPAR $\gamma$ -S112A.

Camp and Tafuri, 1997; Hu et al., 1996). Mice homozygous for the PPAR $\gamma$ -S112A mutation were born at expected Mendelian frequencies, and adipose PPAR $\gamma$  mRNA (not shown) and protein (Figure 1E; see Supplemental Figure S1 at <http://www.developmentalcell.com/cgi/content/full/5/4/657/DC1>) are expressed at levels comparable to wild-type. However, whereas wild-type PPAR $\gamma$  exists in both phosphorylated and nonphosphorylated forms, PPAR $\gamma$ -S112A is exclusively nonphosphorylated (Figure 1E).

PPAR $\gamma$ -S112A mice were healthy and grew at a normal rate, with weights that were not distinguishable from their wild-type littermates (Figure 2A). When challenged with a high-fat diet, the weight gain of PPAR $\gamma$ -S112A mice was not significantly different from wild-type mice (Figure 2A). The total body fat as assessed by dual-energy X-ray absorptiometry (Figure 2B), as well as body fat distribution (not shown), was similar to their wild-type littermates. In addition, no significant change in food intake or oxygen consumption was observed in PPAR $\gamma$ -S112A mice (data not shown).

On normal chow, the glucose tolerance and insulin levels of PPAR $\gamma$ -S112A mice were not distinguishable from those of wild-type mice (Supplemental Figure S2). Remarkably, however, PPAR $\gamma$ -S112A mice were significantly more glucose tolerant than wild-type mice in the

setting of diet-induced obesity (Figure 3A). In this model, PPAR $\gamma$ -S112A mice displayed reduced insulin levels during a 4 hr fast (Figure 3B). Although blood glucose was not significantly different (data not shown), the homeostasis model assessment (HOMA) measure of insulin sensitivity based on simultaneous glucose and insulin determinations was lower in PPAR $\gamma$ -S112A mice, suggestive of increased insulin sensitivity (Figure 3C). Indeed, insulin tolerance testing revealed the PPAR $\gamma$ -S112A mice to be more sensitive to insulin than wild-type (Figure 3D). The PPAR $\gamma$ -S112A mice were studied on a high-fat diet under hyperinsulinemic euglycemic clamp conditions to better understand their preserved insulin sensitivity. Notably, whole-body glucose uptake was ~45% greater in PPAR $\gamma$ -S112A mice (Figure 3E), whereas no significant difference in hepatic glucose production was observed (data not shown). These findings indicate that the insulin sensitivity of PPAR $\gamma$ -S112A mice in the setting of diet-induced obesity was primarily the result of increased whole-body glucose disposal, most quantitatively into muscle.

Adipocyte size correlates inversely with insulin sensitivity, and has been shown to be reduced by TZD treatment (Okuno et al., 1998). Adipocytes from PPAR $\gamma$ -S112A mice were visibly (Figure 4A) and quantitatively (Figure 4B) smaller than wild-type. Because the adipose

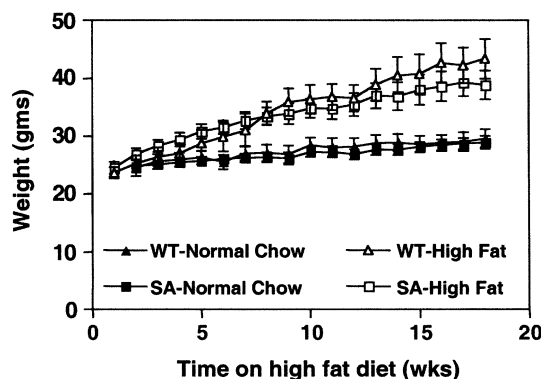
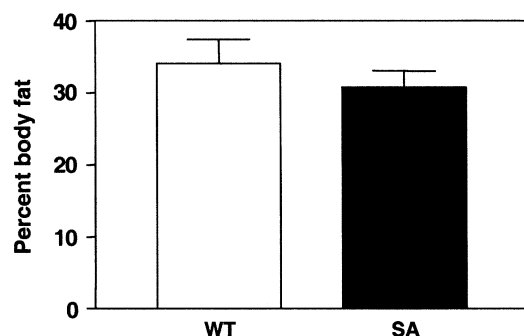
**A****B**

Figure 2. Weight and Body Fat of PPAR $\gamma$ -S112A Mice

(A) Body weights of PPAR $\gamma$ -S112A and wild-type littermate mice on normal chow or on a high-fat diet. A 45% kcal fat diet (Research Diets, D12451) was begun at 5 weeks of age. Shown are means  $\pm$  standard error. On normal chow,  $n = 7$ , PPAR $\gamma$ -S112A;  $n = 9 - 11$ , wild-type; on high-fat chow,  $n = 8$ , PPAR $\gamma$ -S112A;  $n = 7 - 8$ , wild-type.

(B) Percent body fat of control and mutant mice measured by DEXA after 19 weeks on a high-fat diet.  $n = 8$ , PPAR $\gamma$ -S112A;  $n = 7$ , wild-type.

mass was not different, this implies that the total number of adipocytes was increased by the PPAR $\gamma$ -S112A mutation, consistent with its enhanced adipogenic capacity in cultured adipocytes (Hu et al., 1996; Shao et al., 1998) as well as in fibroblasts from these mice (Figure 4C). Along with these changes in adipocyte proliferation and size, PPAR $\gamma$ -S112A mice manifest changes in several serum adipocyte-secreted factors associated with insulin sensitivity. Notably, serum levels of the adipocyte-derived insulin sensitizing hormone adiponectin were significantly increased in PPAR $\gamma$ -S112A mice (Figure 4D). Consistent with this, adiponectin gene expression was increased in adipose tissue from PPAR $\gamma$ -S112A mice (Figure 4E), although the magnitude of the changes in adiponectin gene expression and serum levels was different, as has been observed in other models (Combs et al., 2003). By contrast, serum levels of leptin, which may promote insulin sensitivity (Shimomura et al., 1999), were reduced in PPAR $\gamma$ -S112A mice (Figure 4F). Both the increase in adiponectin and decrease in leptin are consistent with the effects of TZDs (Combs et al., 2002; DeVos et al., 1996; Kallen and Lazar, 1996). Serum levels

of resistin, a TZD-regulated adipocyte hormone whose actions antagonize those of insulin (Rajala et al., 2003; Stepan et al., 2001), were not altered significantly by the PPAR $\gamma$ -S112A mutation (data not shown).

Serum-free fatty acids (FFA) have also been linked to insulin resistance (Boden and Shulman, 2002), and FFA as well as triglyceride levels were significantly lower than in wild-type mice (Figures 4G and 4H). TZDs also reduce FFA levels, increasing flux into white adipose tissue (WAT) by induction of genes such as lipoprotein lipase (LPL) and fatty acid transport protein 1 (FATP1) (Frohnert et al., 1999; Schoonjans et al., 1996), and increasing fatty acid recycling within the adipocyte via induction of PEPCK and glycerol kinase (GyK; Guan et al., 2002; Tontonoz et al., 1995; Tordjman et al., 2003). None of these genes were significantly increased in PPAR $\gamma$ -S112A mice, although there were nonsignificant trends toward induction of adipose FATP1 (WT:  $0.62 \pm 0.14$  [ $n = 4$ ]; S112A:  $2.35 \pm 0.79$  [ $n = 8$ ];  $p = 0.16$ ) and GyK mRNA (WT:  $0.65 \pm 0.09$  [ $n = 4$ ]; S112A:  $1.01 \pm 0.81$  [ $n = 8$ ];  $p = 0.76$ ). The relatively minor changes in expression of TZD-inducible genes involved in adipocyte lipid metabolism may explain why the PPAR $\gamma$ -S112A mutation led to modestly reduced serum FFA but no significant weight gain. Indeed, changes in serum FFA and triglycerides in PPAR $\gamma$ -S112A mice may be related to elevated levels of adiponectin, which have been shown to increase FFA oxidation and predispose to weight loss (Fruebis et al., 2001; Yamauchi et al., 2001b). Although brown adipose tissue (BAT) also expresses PPAR $\gamma$  (Tontonoz et al., 1994), no significant difference in BAT weight, histology, or expression of UCP-1 was observed (Supplemental Figure S3).

We conclude that inheritance of two nonphosphorylatable PPAR $\gamma$  alleles does not produce or exacerbate obesity in mice. Although heterozygosity for a nonphosphorylatable PPAR $\gamma$  allele has been described in four unrelated obese German patients, no genetic linkage has been observed and no additional patients with this allele have been found in the German population (Hamer et al., 2002). The phenotype of the homozygous mutant mice, together with the absence of any genetic linkage or causal connection between the human polymorphism and obesity, cast doubt as to whether heterozygosity for a nonphosphorylated PPAR $\gamma$  variant would be sufficient to cause obesity in humans.

Importantly, however, our data show that elimination of PPAR $\gamma$  phosphorylation protects mice from diet-induced insulin resistance and glucose intolerance. Thus, phosphorylation modulates PPAR $\gamma$  function in vivo. The absence of phosphorylation of PPAR $\gamma$  increases its activity, most likely by increasing its affinity for an endogenous ligand such as  $\Delta 12$ , 14-15-deoxyprostaglandin J<sub>2</sub>, or a related compound (Forman et al., 1995; Kliewer et al., 1995; Shao et al., 1998) and/or increasing the ligand-independent constitutive activity of its N-terminal transactivation domain (Adams et al., 1997; Castillo et al., 1999). Although MAP kinases efficiently phosphorylate PPAR $\gamma$  at serine 112 in vitro and after exposure of cultured adipocytes to growth factors (Camp and Tafuri, 1997; Hu et al., 1996; Shao et al., 1998; Zhang et al., 1996), the identity of the physiological kinase for PPAR $\gamma$  is not known. The phenotype of PPAR $\gamma$ -S112A mice suggests that inactivation of this

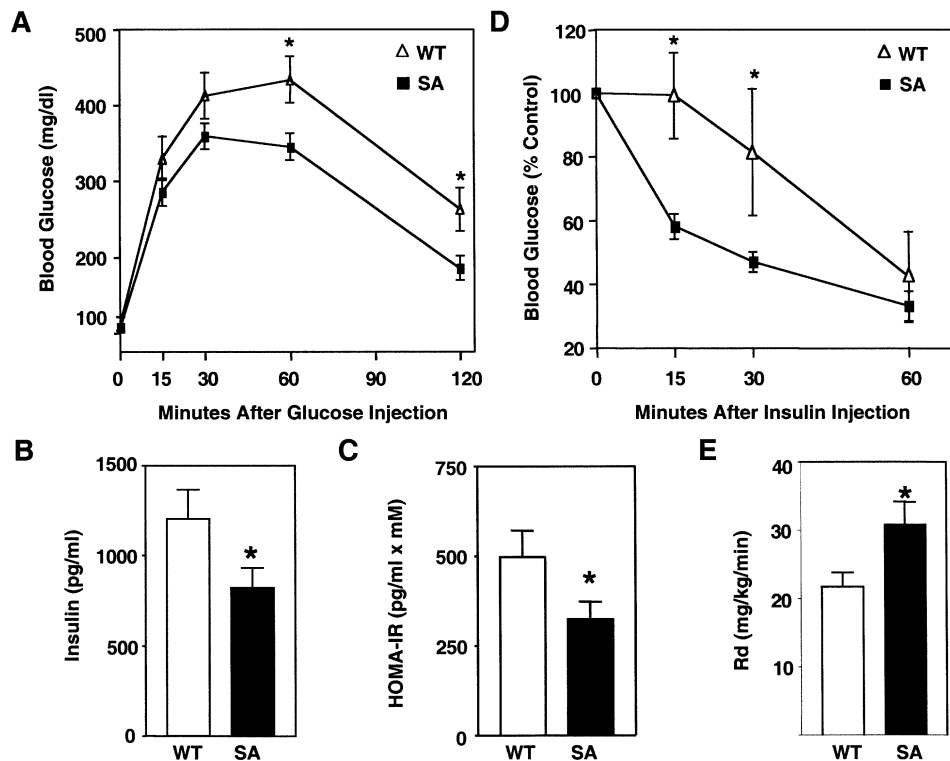


Figure 3. PPAR $\gamma$ -S112A Mice Are Protected from Obesity-Induced Insulin Resistance

(A) Glucose tolerance test. Mice were fed a high-fat diet for 8 weeks prior to testing. Data presented are means  $\pm$  standard error ( $n = 11$ , wild-type;  $n = 19$ , PPAR $\gamma$ -S112A). \* $p < 0.05$ .

(B) Insulin levels of PPAR $\gamma$ -S112A and wild-type mice after a 4 hr fast. \* $p < 0.05$ .

(C) HOMA-IR index of insulin sensitivity (insulin [pg/ml]  $\times$  mM glucose). Serum was collected from mice on a high-fat diet for 5 weeks, fasted for 4 hr beginning at 0800.  $n = 15$ , wild-type;  $n = 22$ , PPAR $\gamma$ -S112A mutants. Data are presented as means  $\pm$  standard error. \* $p < 0.05$  by Student's  $t$  test.

(D) Insulin tolerance test. Mice were fed a high-fat diet for 11 weeks prior to testing. Data presented are means  $\pm$  standard error ( $n = 4$ , wild-type;  $n = 11$ , PPAR $\gamma$ -S112A). For both glucose and insulin tolerance tests, data were analyzed by repeated measures ANOVA followed by posthoc tests using Statview software. \* $p < 0.05$ .

(E) Whole-body glucose uptake. Mice were fed a high-fat diet for 4 weeks, and then hyperinsulinemic euglycemic clamps were performed for 120 min as previously described;  $n = 6$  mice for both groups. Data are presented as means  $\pm$  standard error. Comparisons between experimental groups were performed using two-tailed, unpaired Student's  $t$  test. \* $p < 0.05$ .

kinase would lead to insulin sensitization without excess weight gain.

The inherited preservation of insulin sensitivity without altered adiposity in PPAR $\gamma$ -S112A mice constitutes strong genetic proof that PPAR $\gamma$  contributes directly to glucose homeostasis in vivo. Mice heterozygous for a PPAR $\gamma$  null allele are also insulin sensitive, possibly due to increased leptin levels (Kubota et al., 1999; Yamauchi et al., 2001a). By contrast, the PPAR $\gamma$ -S112A mice have elevated adiponectin levels but reduced leptin levels, suggesting a different mechanism of insulin sensitization. The increased adiponectin in PPAR $\gamma$ -S112A mice is likely to be a direct effect of the mutation in adipose tissue, where PPAR $\gamma$  is most highly expressed. Smaller adipocyte size and reduced serum FFA may also be direct adipocyte effects of nonphosphorylated PPAR $\gamma$ , or could be secondary to the increase in adiponectin. Insulin sensitization by TZDs requires adipose tissue (Chao et al., 2000), and is similarly associated with increased adiponectin, decreased FFA, and smaller adipocytes (Combs et al., 2002; Oakes et al., 1997; Okuno et al., 1998). Like TZD treatment, insulin sensitization

attributable to the nonphosphorylated form of PPAR $\gamma$  is primarily due to increased glucose disposal, most likely to muscle (Inzucchi et al., 1998). Intriguingly, however, TZD-responsive adipocyte genes regulating lipid metabolism such as PEPCK and lipoprotein lipase were not significantly increased in PPAR $\gamma$ -S112A mice. Thus, altered PPAR $\gamma$  activity due to the modulation of its phosphorylation state may be qualitatively different than TZD treatment. As PPAR $\gamma$ -S112A mice are protected from insulin resistance without excess weight gain, compounds that bind to PPAR $\gamma$  to induce the conformation of the nonphosphorylated receptor might have therapeutic value as selective PPAR $\gamma$  modulators, or SPPARMs (Rocchi et al., 2001).

#### Experimental Procedures

##### Generation of Mice

A 129SvEv BAC library was screened by PCR and a PPAR $\gamma$  BAC clone was isolated, mapped by restriction digests, and partially sequenced. The serine 112 residue in exon 2 was mutated to an alanine (S112A), using overlap PCR, and the mutated sequence was used to construct a targeting vector containing exon 2 in the 5'

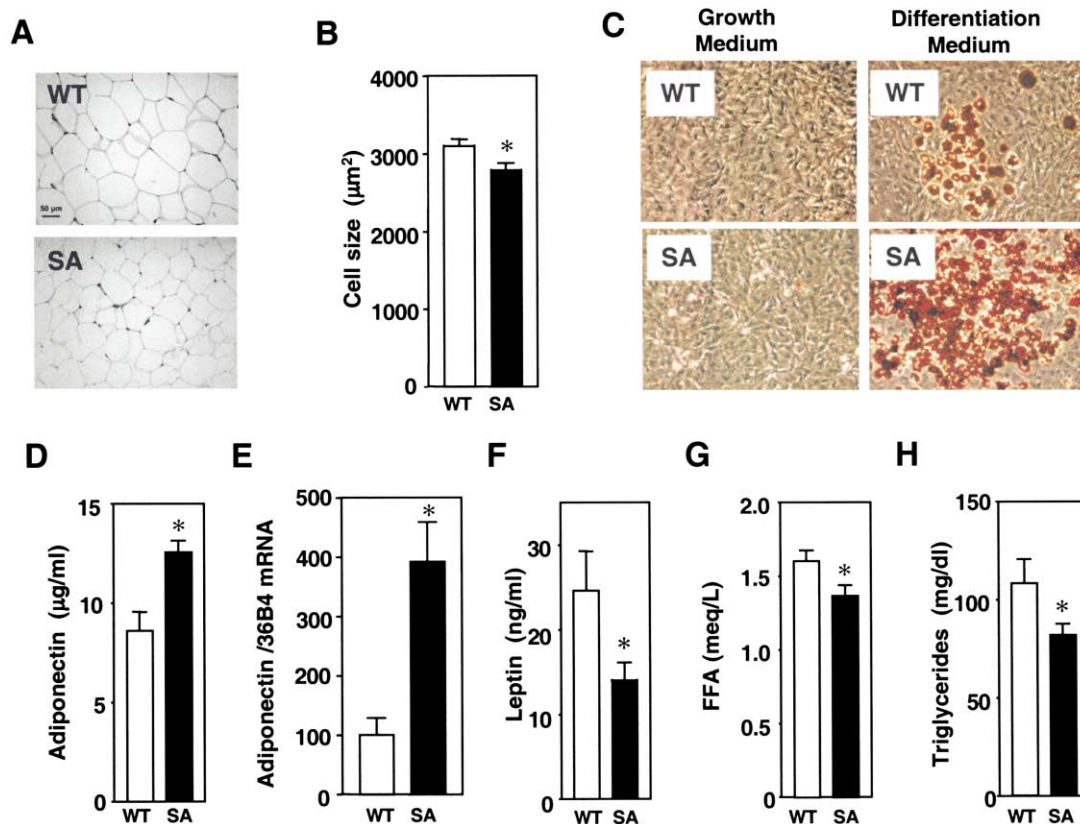


Figure 4. Morphologic and Biochemical Changes in PPAR $\gamma$ -S112A Mice

(A and B) Smaller adipocyte size in PPAR $\gamma$ -S112A mice. Adipocyte morphology (H-E stain).

(B) Adipocyte cell size was measured on dark-field images of paraformaldehyde-fixed sections of epididymal adipose tissue as described in Experimental Procedures. Data are presented as means  $\pm$  standard error. Five hundred-forty adipocytes from three wild-type mice and 610 adipocytes from five PPAR $\gamma$ -S112A mice were analyzed after mice were fed a high-fat diet for 18 weeks. Data were analyzed using unpaired, two-tailed Student's *t* test. \**p* < 0.02.

(C) Increased adipogenesis of MEFs from PPAR $\gamma$ -S112A mice. Oil Red O staining.

(D) Serum adiponectin levels. Mice were fed a high-fat diet for 19 weeks (*n* = 7, wild-type; *n* = 8, PPAR $\gamma$ -S112A).

(E) Adiponectin gene expression in epididymal white adipose tissue, with wild-type normalized to 100% (*n* = 4, wild-type; *n* = 8, PPAR $\gamma$ -S112A). \**p* < 0.005.

(F) Serum leptin levels. Mice were fed a high-fat diet for 3 weeks (*n* = 15, wild-type; *n* = 22, PPAR $\gamma$ -S112A). Shown are means  $\pm$  standard error. \**p* < 0.05, Student's *t* test.

(G and H) Serum-free fatty acid (G) and triglyceride (H) levels. Mice were fed a high-fat diet for 4 weeks (*n* = 15, wild-type; *n* = 22, PPAR $\gamma$ -S112A). Data are presented as means  $\pm$  standard error. Comparisons between experimental groups were performed using two-tailed, unpaired Student's *t* test. \**p* < 0.05.

homology arm. The targeting vector contains a floxed pGK-neo<sup>r</sup> and thymidine kinase (neo-tk) cassette. The targeting vector was introduced into mouse TL-1 embryonic stem cells by electroporation and the cells were subjected to selection under G418. Homologous recombinants were identified using PCR and confirmed by Southern blotting. Targeted lines were expanded and electroporated with a Cre recombinase expression vector to delete the neo-tk cassette. The cells were then subjected to a gancyclovir selection and their genotype was confirmed by PCR as well as by Southern blotting. These clones were injected into blastocysts and resulting male chimeras were mated with female C57Bl6 mice. Germline transmission was confirmed by agouti coat color and PCR. F1 mice were interbred to obtain mice on a mixed 129SvEV/C57BL6 background. These mice were then interbred to obtain mice for experiments. Mice were housed in an environmentally controlled mouse facility with a 12 hr light-dark cycle. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all animal experiments performed. The mice were put on a 45% kcal fat diet (Research Diets, D12451) at the age of 5 weeks. Animals were weighed on a weekly basis. Analysis of body composition was performed using a Piximus dual-energy X-ray absorptiometry scanner (GE).

#### Blood Collection and Serum Measurements

Blood samples were collected from mice between 0900 hr and 1200 hr and were incubated on ice for 30 min before being centrifuged at 20,000  $\times$  g to separate the serum. Serum was stored at  $-80^\circ\text{C}$  before use, and repeated freeze-thaw cycles were avoided on the samples. Serum FFA and triglyceride measurements were performed using kits from Wako Chemicals and Sigma, respectively. Serum insulin and leptin were measured using ELISA kits from CrystalChem. Adiponectin was measured using a radioimmunoassay from Linco Research.

#### Glucose and Insulin Tolerance Tests

For glucose tolerance tests, animals were fasted overnight from 1700 hr to 0900 hr the next day. Basal blood glucose was measured using an Accucheck Advantage glucometer (Roche Diagnostics). Animals were then injected intraperitoneally with a bolus of glucose (2 g/kg), and blood glucose levels were measured at 15, 30, 60, 90, and 120 min. For insulin tolerance tests, animals were fasted for 5 hr, from 0900 hr to 1400 hr. Mice were injected intraperitoneally with a 0.5 U/kg of recombinant human insulin (Humulin), and blood glucose levels were measured at 15, 30, 60, 90, and 120 min.

### Whole-Body Glucose Uptake Studies

Mice were fed a high-fat diet for a period of 4 weeks, after which hyperinsulinemic euglycemic clamps were performed for 120 min as previously described (Kim et al., 2001).

### Adipocyte Morphology Studies

Dark-field images of paraformaldehyde-fixed sections of epididymal adipose tissue were quantitated as described (Chen and Farese, 2002), except IP Lab software was used (Scanalytics).

### MEF Adipogenesis Assay

Day 13.5 embryos were homogenized by passage through a 181/2 gauge needle, and then plated out on a 10 cm tissue culture dish and passaged when confluent. Experiments were performed on cells that were passage 4–8. Two days past confluence, cells were incubated with DMEM containing 10% FBS, insulin (10  $\mu$ g/ml), isobutylmethylxanthine (0.5 mM), dexamethasone (1  $\mu$ M), and rosiglitazone (1  $\mu$ M). This was considered day 0 of differentiation. On day 2 and every 2 days thereafter, the medium was changed to DMEM containing 10% FBS, insulin (10  $\mu$ g/ml), and rosiglitazone (1  $\mu$ M). The experiments were ended at day 7, and cells were harvested for RNA or stained with Oil Red O.

### Gene Expression Studies

RNA from tissues was isolated using Trizol reagent (Life Technologies). Northern blots were performed as described (Chawla et al., 1994).

### Statistical Analysis

Data are presented as means  $\pm$  SEM. Comparisons between experimental groups were performed using two-tailed, unpaired Student's *t* test. In the case of glucose and insulin tolerance tests, data were analyzed by repeated measures ANOVA followed by posthoc tests using Statview software.

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